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(54) Title: METHOD OF TREATING COLLECTED MAMMAL SEMEN AND SEPARATING SPERM INTO X AND Y COMPONENTS

(57) Abstract

A method of sexing viable sperm cells using a cell cytometer in combination with selective labelling of sperm cells with fluorescent dye. Upon excitation of the selectively labelled X and Y chromosome bearing cells a detection system is activated so that X and Y sperm cells are channeled into two separate collection ports.

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Method of Treating Collected Mammal Semen and Separating Sperm Into X and Y Components

Technical Field

This invention relates to an improved method of preserving collected mammalian semen and separating the sperm contained therein into X and Y components so that a female of the species may be artificially inseminated with separated sperm to produce offspring of a desired and predetermined sex. This invention is particularly useful for preserving cattle semen and separating it so that sperm bearing only X chromosomes can be separated and used to produce dairy herds and sperm bearing only Y chromosomes can be used to produce beef herds. This invention also has usage in breeding horses and other animals.

Background Art

Until now, no known way has been suggestd in the prior art for obtaining large populations of viable sexed sperm. This is of particular significance in cattle breeding wherein it is desired to have a large number of female offspring when breeding dairy cattle and a large number of male offspring when breeding beef cattle. Also, such a sexing technique, if available, would have wide application in many situations for example involving humans where predetermination of sex of offspring would be desirable.

Some physical characteristics of sperm that are of potential use in separating sperm cells are: higher density or packing of the Y chromosome, greater DNA content of the X chromosome, slightly larger size and heavier weight of sperm containing the X chromosome; generally higher velocity of movement of the Y chromosome containing sperm; and an apparent dense negative electrical surface charge on the X chromosome. However, the methods of separation of sperm utilizing these physical properties have, for the most part, been only partially successful, with separation percentages ranging from 60% to 80%. Thus, there is a

very great need for a method of sperm separation which is virtually 100% accurate and which can be done very rapidly. Furthermore, in order to accomplish separation, it is necessary to maintain or get the semen which has been collected in a fluid non-coagulated condition before the separation of the sperm can be accomplished.

It is well known that in primates, such as humans and gorillas, the Y chromosome of sperm tends to fluoresce to a special brightness when stained with a dye such as quinicrine or quinicrine mustard whereas the X chromosome of sperm will not so fluoresce. It is also known that the sperm of most other animals will not normally be subject to this selective staining unless a cell membrane diffusion material or agent, such as an appropriate enzyme or chemical, is used to facilitate passage through the cell membrane so that the dye can enter the cell. A commercially available chemical for this purpose is dimethylsulfoxide. It is also known that by adding papaya protease to the sperm of horses and bulls, the cell membrane can be penetrated by the dye so that selective identification of the X and Y chromosomes can be accomplished under a microscope. A disclosure of this technique is found in U.S. Patent No. 4,155,831 entitled "Thermal Convection Counter Streaming Sedimentaion and Forced Convection Galvanization Method and Apparatus for Controlling the Sex of Mammalian Offspring," issued May 22, 1979 to Bhairab C. Bhattacharya. Such a process has been used in the prior art to determine the success ratio in separating X and Y chromosome bearing sperm, hereinafter X and Y sperm, by other known methods.

A device for identifying and then separating live blood cells from dead blood cells is disclosed in U.S. Patent No. 3,791,517 for "Digital Fluid Amplifier Particle Sorter," issued February 12, 1974 to Mitchell Friedman. It is suggested in this patent that the separation of sperm cells be done, by use of selective fluorescent radiation responses from X and Y sperm labelled differentially with a fluorescent dye.

Disclosure of Invention

In accordance with this invention, a method of separating viable sperms cells according to whether they carry an X chromosome or a Y chromosome is described. The separated batches of cells are suitable for use in artificial insemination of humans, cows and other livestock animals so that offspring of predetermined sex are obtained.

The method of separation, it is hypothesized, depends on basic differences in X and Y sperm cells. For example, each X sperm has a greater DNA content then a corresponding Y sperm since the X chromosome has a greater DNA content than a comparable Y chromosome. Hence, it is postulated that the X sperm should incorporate greater absolute amounts of certain dyes and other labels then Y sperm. On the other hand, it is believed that the Y chromosome of Y sperm is more dense or tightly packed than a corresponding X chromosome. Hence it is postulated some dyes and other label should become incorporated or associated with the Y chromosome in a denser fashion than they do for a comparable X chromosome and will be perceived accordingly.

Semen which contains sperm is collected from a mammal and treated by adding an anti-coagulant, such as mammal saliva, to prevent coagulation and hasten liquefaction. Any anticoagulant that does not affect viability will work. In particular, human saliva has been found very satisfactory for this purpose and is readily available. The semen is then diluted with a diluting solution and stored in a water bath at substantially the body temperature of the mammal or the sperm cells are centrifuged out of the semen fluid and diluted in a suitable saline solution.

When the semen is of mammals other than primates and voles, a cell membrane diffusion material, such as sperm-free human semen, can be added to the mammalian semen so that a fluorescent dye can penetrate the cell membrane of the sperm cell and become localized around the chromosomes therein. The dye will penetrate the cell membranes of the sperm cell of primates and vole semen without prior preparation.

The selection of dye depends on the method of detection used to select the X and Y sperm. If a scanning procedure is used wherein any bright spots of fluorescent dye would be detected, such as that which it is postulated characterize the Y chromosome because of its higher density than an ideal dye is quinicrine or quinicrine mustard.

However, if the total fluorescence per sperm cell is being detected then a more suitable dye has been found to be acridine, acridine orange and derivatives thereof such as ethidium, bromide, mithramycin or any combination thereof. Another suitable dye is DAPI (4, 6-diamidino-2-phenylindole).

Following staining with the dye the semen is put into a detection chamber. The semen is flowed through the chamber so that ideally only one sperm cell at a time is irradiated with ultraviolet light. The light causes the dye to fluorescence. A detection devise in the chamber senses the degree of fluorescence and provides an output signal in response to the sensed cell which varies according to the degree of fluorescence. The stream then passes through a fluidic amplifier and the stream is switched in response to each sensed cell in the detection chamber to direct the sensed cell to one of two outlet ports. If the detection device is programmed to scanning the cell for bright spots the Y sperm will trigger the system presumably because of the higher density of the dye localized around the denser Y chromosome. If instead the greater absolute degree of fluorescence is being detected, the X sperm will trigger the system presumably because the X sperm contains more DNA; and, hence more dye is localized around it. In any way, the X and Y chromosomes can be effectively separated into separate containers for use in artificial insemination of female animals in order to produce the desired sexed offspring.

It will be apparent that this method of treating mammalian semen and separating X and Y sperm is rapid and highly efficient.

Additional advantages of the invention will become apparent from the detailed description which follows.

Best Mode for Carrying Out the Invention

Semen is first collected from a mammal in any manner which is known to one skilled in the art. For example, with cattle and horses, teaser animals or electrical stimulation may be used. The various methods of collecting semen from animals is explained in conventional vetenarian reference texts.

The collected semen is placed in a clean, sterile container and may be treated with an enzyme solution to hasten liquefaction. Cattle semen for example is highly viscous and will not flow in a stream as necessary for separation unless an anti-coagulant is added and a dilutant is also added. Advantageously, it has been found that one of the best enzyme solutions and one of the most physiologically compatible is mammalian saliva, and particularly human saliva. In practice, about 1 ml of human saliva is added to each quantity of about 2.0 ml to about 3.5 ml of semen. If the quantity of human saliva added is significantly less than 1 ml, the reaction will be too slow. However, an amount larger than 1 ml will have no adverse effect, but is unnecessary.

The semen is then diluted with any known commercially available semen diluting solution on about a 1:1 basis. Commercially available semen diluting solutions contain egg yolk, glycerol, glucose and citrate. Preparation of these solutions is set forth in Fertility and Sterility by Berman and Sasada, published in 1966. One such anti-coagulant is alpha-amylase. The semen is then stored in a water bath at approximately the body temperature of the animal. This is between about 35°C and about 38.5°C, but is usually about 37°C. If the temperature is substantially above or below this range, sperm mobility will decrease and the survival rate of the sperm will decrease below acceptable limits.

When it is time to selectively identify and separate the X and Y sperms and the mammal is not a primate or a vole, a cell membrane diffusion material must be added to the collected semen so that the dye can penetrate the cell. The dye will penetrate in primate and vole semen without further treatment. One cell

membrane diffusion material which has been found satisfactory is DMSO, commonly known as dimethylsulfoxide and sometimes referred to as disulfomethoxide. It has been found that the addition of about 0.05 ml of DMSO to a quantity of about 2.0 ml to about 3.5 ml of semen provides sufficient penetration of the cell membrane by the dye.

Another cell membrane diffusion material which has been found quite satisfactory is sperm-free human semen. The sperm-free human semen is added in a quantity of about 0.5 ml human semen to a quantity of about 2.0 ml to about 3.5 ml of mammal semen. If either DMSO or human semen is added in quantities substantially below the indicated ranges, the diffusion of the dye will be very slow whereas if the amounts added are substantially above the indicated ranges, the DMSO and human semen will have a toxic effect and kill substantial numbers of sperm.

The dyes which has been found most satisfactory are taken from the group consisting of quinicrine, quinicrine hydrochloride, quinicrine dihydrochloride and quinicrine mustard. When quinicrine hydrochloride or quinicrine dihydrochloride is used, it is diluted in a solution of ion-free distilled water to a strength in the range of about 0.05% to about 5.0%. The most satisfactory range has been found to be between about 0.5% and about 1.0% in solution. When quinicrine mustard is used, it is diluted with ion-free distilled water to a solution having a strength in the range of about 0.001% to about 0.01% whereas the optimum range is about 0.005% solution. If the solutions are substantially below the indicated range in concentration, the dye will take too long to stain, whereas if the solutions are substantially above the indicated ranges in concentration, the dye will have a toxic effect and kill substantial numbers of the sperm. The dye solution is put in contact for a period of at least several minutes, but usually for a period of at least 15 minutes to assure good dye penetration. Confirmation of staining is obtained by separating a small part of the specimen and checking for staining under a fluorescent microscope.

After the semen has been thus treated, it can be fed through a digital fluidic amplifier particle sorter such as that described in the above-mentioned U.S. Patent No. 3,791,517 to Friedman. This device is sold under the trademark "CYTOFLUOROGRAF" by Ortho Instruments, Inc. of Westwood, MA, U.S.A. The disclosure of this patent is incorporated herein by reference. By using this apparatus, the difference in fluorescence of X and Y chromosome bearing cells entrained in a stream of diluted semen is detected and the resultant different signals which are produced as a result of this detection are used to control a fluidic amplifier located downstream to switch the X sperm to one outlet port and the Y sperm to another outlet port so they can be separated and collected.

In particular, the semen is fed in a very narrow stream so that the sperm cells in the semen move essentially single file past a laser light beam which is provided with an ultraviolet filter. The laser projects a very narrow beam in which a pattern of illumination of the beam, when it strikes the sperm cell, appears as a thin line of light transverse to the stream of cells. Electrical photoresponsive pick-up elements are arranged around the outside of the detection chamber through which the stream passes to detect any scattering of light due to the ultraviolet light striking a fluorescent Y chromosome. The excitation wavelength of the laser beam is in the range of 457 nm to 488 nm. The emission wavelength from the fluorescent Y sperm will be around 351 nm. The fluid amplifier includes a transducer which is responsive to any scattering of light due to fluorescence of a Y sperm which it is postulated will create a turbulance in the stream to cause a "wall attachment effect" as is well known in fluidics. Thus, any X chromosome it is postulated will pass along the stream uninterrupted and out a first outlet port whereas the Y chromosomes will be diverted due to reaction of the transducer to the scattered light causing them to be diverted and pass through a second outlet port. By this means, the semen can be separated into X and Y sperm and separately collected for

artificial insemination of an animal at a future time wherein the sex of the offspring will be predetermined. The separation accuracy of this method is extremely high compared with the 60% to 80% separation possible with other techniques. In practice, using this technique, the separation effectiveness is nearly 100% and is at least greater than 90%. After separation, the sperm may be frozen for use at a future time in a manner which is well known.

From the foregoing, it can be seen that a highly novel and efficient method has been provided for preparing mammal semen for separating the X and Y sperm so that a female animal such as a cow or horse can be artificially inseminated and the sex of the offspring predetermined. Of course, it will be understood that the use of this technique would have application with respect to any mammal.

The invention has been described in detail with particular reference to a preferred embodiment thereof, but it will be understood that variations and modifications can be effected within the spirit and scope of the invention.

Claims

What is claimed is:

1. A method of preserving semen for use in artificial insemination comprising the steps of:

collecting semen from a mammal;

treating the mammal semen by adding mammal saliva to prevent coagulation;

storing the semen in a water bath at substantially the body temperature of the mammal.

2. The method, as claimed in Claim 1, wherein:

human saliva is added to the mammal semen to prevent coagulation.

3. The method, as claimed in Claim 1, wherein:

a quantity of about 1.0 ml of human saliva is added to a quantity of about 2.0 ml to about 3.5 ml of mammal semen.

4. The method, as claimed in Claim 1, wherein:

the mammal semen is diluted on about a 1:1 ratio.

5. The method, as claimed in Claim 1, wherein:

the treated and diluted mammal semen is stored at a temperature in the range of about 35°C to about 38.5°C.

6. The method, as claimed in Claim 4, wherein:

the treated and diluted semen is stored at a temperature of about 37°C.

7. A method of separating viable X and Y chromosomes bearing sperm cells comprising the steps of:

(a) selectively staining the chromosomes of the sperm cells with a flurescent dye;

(b) flowing the stained sperm cells, single file, through a flow cytometer comprising a detection chamber;

(c) irradiating said sperm cells with ultraviolet light;

(d) detecting fluorescence;

(e) translating the detected fluorescence into an output signal;

(f) switching the flow in response to the output signal;

(g) collecting the sexed sperm.

8. The method, as defined in Claim 7 wherein the detection chamber detects total DNA fluorescence.

9. The method as defined in Claim 8 wherein the fluorescent dye is selected from the group consisting of acridine, acridine orange and derivatives thereof.

10. The method as defined in Claim 9 wherein the dye is ethidium bromide and mithramycin.

11. The method as defined in Claim 7 wherein the detection chamber detects the fluorescence of the Y chromosome.

12. The method as defined in Claim 11 wherein the fluorescent dye is selected from the group consisting of quinicrine, quinicrine hydrochloride, quinicrine dihydrochloride, and quinicrine mustard.

13. The method, as claimed in Claim 12, wherein,

the dye is a quinicrine solution having a strength of about 0.05% to about 5.0% of quinicrine hydrachloride and quinicrine dihydrochloride.

14. The method, as claimed in Claim 13, wherein,

said solution has a strength of about 0.5% to about 1.0%.

15. The method, as claimed in Claim 11, wherein,

the dye is a quinicrine mustard solution having a strength of about 0.001% to about 0.01% or quinicrine mustard.

16. The method, as claimed in Claim 15, wherein,

said solution has strength of about 0.005%.

17. The method, as claimed in Claim 11, wherein,

said semen is irradiated with ultraviolet light having a wavelength between about 457 nm and about 488 nm.

18. A method of separating X and Y sperm in mammal semen comprising the steps of:

collecting semen from the mammal;

adding an anticoagulant to prevent coagulation;

diluting the semen;

maintaining the temperature of the treated and diluted semen at a temperature of about 37°C;

adding a flurescent dye to the semen to cause the Y chromosome to specially fluoresce under ultraviolet light;

irradiating the semen with ultraviolet light;

moving the semen in a stream through a detection chamber;

sensing the presence of the specially fluorescent Y chromosome in the detection chamber;

providing an output signal in response to the sensed chromosome;

passing the stream from the detection chamber through a fluidic amplifier; and

switching the stream in response to the Y chromosome to direct the Y containing sperm cell to one of two outlet ports, and the X chromosome containing sperm cell to the other outlet port collecting the sexed sperm.

19. The method, as claimed in Claim 18 wherein,

about 0.05 ml of DMSO is added to about 2.0 ml to about 3.5 ml of semen as the cell membrane diffusion material.

20. The method, as claimed in Claim 18, wherein,

about 0.5 ml of sperm-free human semen is added to about 2.0 ml to about 3.5 ml of semen as the cell membrane diffusion material.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US82/01478

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC 3

A01N 1/02 435/2
A61K 35/48 424/105

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/2 209/577, 580, 587 424/105 436/63, 906

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

Chemical Abstracts 8th Collective to date
"semen", "sperm", "saliva"

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
A	US, A, 3,185,623 Published 25 May 1965, Smith et al	1-6
X	US, A, 3,586,859 Published 22 June 1971, Katz et al	7-20
A	US, A, 3,816,249 Published 11 June 1974, Bhattacharya	1-6
A	US, A, 4,092,229 Published 30 May 1978, Bhattacharya	7-20
X	US, A, 3,207,910 Published 21 September 1965 Hirschfeld et al	7-20
X	US, A, 3,305,089 Published 21 February 1967 Fraenkel	7-20

* Special categories of cited documents: [15]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
15 February 1983	02 MAR 1983
International Searching Authority [1]	**Signature of Authorized Officer [10]**
ISA/US	Sam Rosen SAM ROSEN EXAMINER

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